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Evaluation of Immunophenotypic and Molecular Biomarkers for Sézary Syndrome Using Standard Operating Procedures: A Multicenter Study of 59 Patients

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Differentiation between Sézary syndrome and erythrodermic inflammatory dermatoses can be challenging, and a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could be useful as additional diagnostic criteria. In this European multicenter study, the sensitivity and specificity of these immunophenotypic and recently proposed but unconfirmed molecular biomarkers in Sézary syndrome were investigated. Peripheral blood CD4⁺ T cells from 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses were analyzed for cell surface proteins by flow cytometry and for copy number alterations and differential gene expression using custom-made quantitative PCR plates. Experiments were performed in duplicate in two independent centers using standard operating procedures with almost identical results. Sézary cells showed *MYC* gain (40%) and *MNT* loss (66%); up-regulation of *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%), and *PLS3* (66%); and down-regulation of *STAT4* (91%). Loss of CD26 ($\geq 80\%$ CD4⁺ T cells) and/or CD7 ($\geq 40\%$ CD4⁺ T cells) and combination of altered expression of *STAT4*, *TWIST1*, and *DNM3* or *PLS3* could distinguish, respectively, 83% and 98% of patients with Sézary syndrome from patients with erythrodermic inflammatory dermatoses with 100% specificity. These additional diagnostic panels will be useful adjuncts in the differential diagnosis of Sézary syndrome versus erythrodermic inflammatory dermatoses.

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Abbreviations: CNV, copy number variation; EID, erythrodermic inflammatory dermatoses; GE, gene expression; PBMC, peripheral blood mononuclear cell; SOP, standard operating procedure; SS, Sézary syndrome

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INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma that is derived from CD4⁺ skin-homing memory T cells and characterized by erythroderma, generalized lymphadenopathy, and neoplastic T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood (Wieselthier and Koh, 1990).

Differentiation between SS and erythrodermic inflammatory dermatoses (EID) may be extremely difficult, both clinically and histopathologically, but is very important from therapeutic and prognostic perspectives. For a long time the diagnosis was based on demonstration of atypical T cells, so-called Sézary cells, in blood smears (Sentis et al., 1986; Trotter et al., 1997). However, it was shown that Sézary cells can also be observed in the peripheral blood of patients with EID and even in healthy control subjects (Duncan and Winkelmann, 1978; Meijer et al., 1977). Demonstration of at least 1,000 Sézary cells per mm³ was often used as a decisive criterion, but this was not generally agreed on (Wieselthier and Koh, 1990). To prevent patients with EID

being misclassified as having SS and being treated as such, in 1997 the European Organization for Research and Treatment of Cancer group proposed the demonstration of clonal T cells and the presence of an expanded CD4⁺ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS (Willemze et al., 1997).

At present, the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in the skin), in combination with one or more of the following criteria: an absolute Sézary cell count greater than 1,000 cells per mm³; loss of T-cell markers CD2, CD3, CD4, and/or CD5; and/or an expanding population of CD4⁺ T-cells leading to a CD4/CD8 ratio of more than 10 (Swerdlow et al., 2008; Willemze et al., 2005). However, distinction between SS and EID can still be difficult, because T-cell clonality can be observed in a substantial proportion of patients with EID as well, and not all SS patients have a CD4/CD8 ratio of greater than 10 at first presentation (Vonderheid, 2006; Vonderheid and Bernengo, 2003).

To solve this diagnostic problem, a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells. Flow cytometry studies reported loss of CD7 and CD26 expression by Sézary cells and suggested CD4⁺CD7[−] cells of at least 40% and CD4⁺CD26[−] cells of at least 30% as tentative diagnostic criteria in those difficult cases (Bernengo et al., 2001; Fierro et al., 2008; Harmon et al., 1996; Jones et al., 2001; Kelemen et al., 2008; Klemke et al., 2008; Lima et al., 2003; Olsen et al., 2007; Rapp et al., 2001; Sokolowska-Wojdylo et al., 2005; Vonderheid et al., 2002; Vonderheid and Bernengo, 2003; Washington et al., 2002).

In addition, recent studies described expression of killer cell immunoglobulin-like receptors CD158a, CD158b, and CD158k and the “central memory” T-cell phenotype (CD27⁺, CD45RA[−], CD45RO⁺) as characteristic features of Sézary cells (Bagot et al., 2001; Bahler et al., 2008; Campbell et al., 2010; Dummer et al., 1996; Fierro et al., 2008; Karenko et al., 2001; Klemke et al., 2008; Lima et al., 2003; Marie-Cardine et al., 2007; Michel et al., 2013; Poszepczynska-Guigne et al., 2004). Molecular investigations identified gain of *JUNB*, *MYC*, and loss of *MYC* antagonists *MNT* and *MXI1* as recurrent genetic lesions in the SS genome (Mao et al., 2003; Mao et al., 2008; Vermeer et al., 2008). Gene expression studies showed increased expression of *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *JUNB*, *TWIST1*, *EPHA4*, and *MYC* and decreased expression of *STAT4* in Sézary cells (Booken et al., 2008; Goswami et al., 2012; Kari et al., 2003; Mao et al., 2008; Nebozhyn et al., 2006; Su et al., 2003; Van Doorn et al., 2004).

However, the diagnostic value of these biomarkers in diagnosing SS has not been investigated thoroughly. Moreover, most biomarkers were identified in small, single-center studies with a limited number of patients and controls and have not been confirmed in large independent studies. In addition, flow cytometry studies have used widely differing protocols, which impedes interpretation and comparison of results from different studies.

The goal of this European Organization for Research and Treatment of Cancer multicenter study was to investigate the sensitivity and specificity of these biomarkers for SS in a large group of well-defined SS patients compared with EID patients using standard operating procedures (SOPs).

RESULTS

Patient characteristics

Clinical characteristics at diagnosis of the 59 SS patients and 19 EID patients are summarized in Table 1.

The patients with SS had a T-cell clone in the peripheral blood (59 of 59), a CD4/CD8 ratio above 10 (53 of 57), and/or a Sézary cell count above 1,000 per mm³ (34 of 43, including all four patients with a CD4/CD8 ratio lower than 10).

One EID patient showed a T-cell clone in the peripheral blood, and another EID patient had a CD4/CD8 ratio above 10 because of very low numbers of CD8⁺ T-cells, but none had a Sézary cell count above 1,000 mm³ (Table 1).

Flow cytometry

Flow cytometry experiments were performed both in Leiden and Paris for all 59 SS patients and 19 EID patients. Differences in flow cytometry results between Leiden and Paris were greater than 20% in 99.8% of individual assays, and in these cases an average was used in further analysis. In only 2 of 1,027 assays (0.2%) did the differences in results exceeded 20%, and these were therefore excluded from further analysis.

In this study, 87% of SS patients (46 of 53) had a CD4/CD8 ratio above 10 at inclusion, compared with 8% of the EID patients (1 of 12) (sensitivity = 87%, specificity = 92%).

The CD4⁺ gated lymphocytes were CD3⁺ and CD8[−]. In the CD4⁺ T-cell population, 7 of 59 (12%) SS patients showed loss for CD2 (median = 45%, range = 32–100%) and 4 (7%) patients showed diminished expression for CD2 (CD2^{dim}), whereas this was never observed in the 19 EID patients. One SS patient showed 90% CD5 loss, compared with none of the EID patients.

In the CD4⁺ T-cell population, a percentage of CD4⁺CD7[−] cells above 40% was found in 32 of 59 (54%) SS patients but

Table 1. Patients' clinical characteristics at diagnosis

Characteristic	SS Patients (n = 59)	EID Patients (n = 19)
Male:female ratio	37:22	16:3
Median age (range), years	65 (32–89)	67 (29–86)
Erythroderma	46/52 (88)	19/19 (100)
Pruritus	45/52 (87)	13/19 (68)
Ectropion	6/52 (12)	1/19 (5)
Hyperkeratosis hand/feet	21/52 (40)	3/19 (16)
Palpable lymphadenopathy	21/52 (40)	4/19 (21)
Lymphadenopathy confirmed by CT scan	17/41 (41)	1/7 (14)
Leukocytes $\geq 10.0 \times 10^9/L$	40/56 (71)	5/14 (36)
CD4/CD8 ratio ≥ 10.0	53/57 (93)	1/12 (8)
Absolute Sézary cell count $\geq 1,000 \text{ mm}^3$	34/43 (79)	0/10 (0)
T-cell clone in peripheral blood	59/59 (100)	1/17 (6)
Identical T-cell clone in blood and skin	32/38 (84)	0/3 (0)

Note. Values are given as n/total (%) unless otherwise noted.
Abbreviations: CT, computed tomography; EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.

never in EID patients (sensitivity = 54%, specificity = 100%). In addition, CD7^{dim} was found in 2 of 59 (3%) SS patients, compared with none of the EID patients.

In the CD4⁺ T-cell population a percentage of 30% or more CD4⁺CD26⁻ cells was found in 51 of 59 (86%) SS patients but also in 10 of 19 (53%) EID patients (sensitivity = 86%, specificity = 47%). When shifting the percentage to 80%, 39 of 59 (66%) SS patients but none of the EID patients had CD4⁺CD26⁻ cells of 80% or more in the CD4⁺ T-cell population (sensitivity: 66%; specificity: 100%). In addition, CD26^{dim} was found in 5 of 59 (8%) Sézary patients and in one of 19 (5%) EID patients.

Loss of CD26 by more than 80% and/or loss of CD7 by more than 40% of CD4⁺ T cells was found in 49 of 59 (83%) SS patients but was never observed in the EID patients (sensitivity = 83%, specificity = 100%).

Investigations on CD158k expression showed that more than 5% of these CD4⁺ T cells expressed CD158k or CD158k^{low} in 19 of 58 (33%) SS patients, compared with 1 of 19 (5%) EID patients (sensitivity = 33%; specificity = 95%). The results (including expression of CD158a and CD158b) are summarized in Table 2.

No major difference was observed in the expression of CD27, CD45RA, and CD45RO by CD4⁺ T cells between SS and EID patients (data not shown).

Copy number variation

Copy number variation (CNV) experiments were performed in 58 SS patients and 17 EID patients in Leiden. Duplicate experiments for 14 samples were performed in London, which gave identical results (see Supplementary Figure S1 online).

In 47 of 58 (81%) SS patients, alterations in copy number were found compared with none of the 17 EID patients. Gain of *MYC* was observed in 23 of 58 (40%) SS patients (sensitivity = 40%, specificity = 100%). *MNT* loss was found in 38 of 58 (66%) SS patients (sensitivity = 66%, specificity = 100%) and one (2%) patient showed gain of *MNT* (Figure 1). Gain of *MYC* and/or loss of *MNT* was found in 76% (44 of 58) of SS patients (sensitivity = 76%, specificity = 100%). Copy number alterations of *JUNB* and *TWIST1* were found in only a minority of SS patients (Figure 1).

Gene expression

Gene expression (GE) analysis were performed on 55 SS, 19 EID, and 4 healthy control patients in Leiden. Thirty samples

were also analyzed in London, with identical results in 28 samples (see Supplementary Figure S2 online); two samples could not be analyzed because of a technical error.

DNM3, *TWIST1*, *EPHA4*, *PLS3*, and *STAT4* were the most differentially expressed genes in SS patients compared with EID patients and healthy controls with 100% specificity ($P < 0.001$) (Table 3, see Supplementary Figure S3 online). Up-regulation of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3* was found in 66–75% of SS patients, and *STAT4* was down-regulated in 91% of SS patients (Figure 2). Up-regulation of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3* was found in 41 of 55 (75%), 38 of 55 (69%), 36 of 55 (66%), 36 of 55 (66%) of SS patients, respectively, and *STAT4* was down-regulated in 50 of 55 (91%) of SS patients. Combining alterations in gene expression (*STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3*), we could distinguish 54 of 55 (98%) SS patients from all EID patients (sensitivity = 98%, specificity = 100%).

Aberrant gene expression of *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, and *JUNB* was found in only a minority of SS patients (Table 3).

DISCUSSION

In the present multicenter study, we investigated the diagnostic sensitivity and specificity of immunophenotypic and molecular biomarkers in SS using SOPs. We show that by using SOPs, it is possible to obtain highly reproducible results for flow cytometry, CNV, and GE analysis and show that loss of CD7 and CD26 by CD4⁺ T cells; gain in copy number of *MYC* and loss of *MNT*; increased expression of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3*; and decreased expression of *STAT4* are highly characteristic for Sézary cells.

In the current study most SS patients have a CD4/CD8 ratio above 10, but we also show that a significant minority of patients (13%) does not fulfill this diagnostic criterion. For these patients additional immunophenotypic markers are clearly needed.

Comparison of results from previous flow cytometry studies is hindered by the use of different protocols and cell populations. In the present study we focused on CD4⁺ gated T cells because CD4 is rarely lost by Sézary cells, facilitating the comparison of expression levels of different immunophenotypic markers.

Previous studies reported that a CD4⁺CD26⁻ cell population greater than 30% had a sensitivity of 97% and specificity of 100% in diagnosing peripheral blood involvement

Table 2. Overview of the tested flow cytometry markers in 59 SS and 19 EID patients at inclusion of the study

Markers for SS Described in the Literature	SS Patients, n/total (n = 59)	EID Patients, n/total (n = 19)	Sensitivity, %	Specificity, %
CD4/CD8 ratio ≥ 10	46/53	1/12	87	92
CD4 ⁺ CD7 ⁻ $\geq 40\%$	32/59 ¹	0/19	54	100
CD4 ⁺ CD26 ⁻ $\geq 30\%$	51/59 ²	10/19	86	47
CD158a ³	2/58	0/19	3	100
CD158b ³	13/59	1/19	22	95
CD158k ³	19/58	1/19	33	95

Abbreviations: EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.

¹Including four of seven SS patients with a CD4/CD8 ratio below 10.

²Including five of seven SS patients with a CD4/CD8 ratio below 10.

³Low expression and expression of 5% or more of the CD4⁺ lymphocytes.

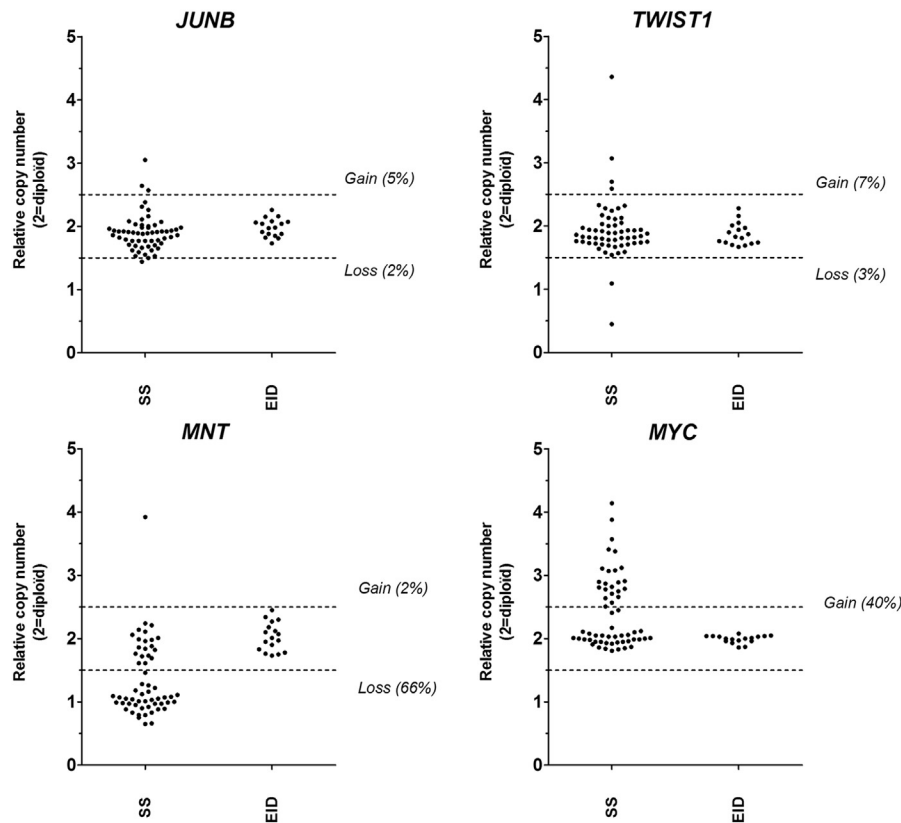


Figure 1. Copy number variation results for *JUNB*, *TWIST1*, *MNT*, and *MYC*. The gains and losses in copy number in 58 Sézary syndrome compared with 17 erythrodermic inflammatory dermatoses patients are shown as normalized relative copy number, where 2 represents diploid DNA. The dotted lines signify the chosen thresholds for gain and loss, 2.5 and 1.5, respectively.

and suggested this cut-off point as a tentative diagnostic criterion for SS (Bernengo et al., 2001; Vonderheid and Bernengo, 2003). Indeed, loss of CD26 in more than 30% of CD4⁺ T cells was found in 86% of SS patients but also in 53% of EID patients, resulting in a specificity of 47%. However, when using a percentage of 80% as the cut-off point, we found that 39 of 59 (66%) SS patients but none of the EID patients met this criterion.

Table 3. Results of aberrant gene expression in all tested genes in 55 SS patients relative to 19 EID patients and 4 healthy control subjects¹

Gene (Up-Regulation or Down-Regulation)	SS, n/total (n = 55)	Sensitivity, %
<i>PLS3</i>	36/55	66
<i>DNM3</i>	41/55	75
<i>CDO1</i>	20/55	36
<i>TRAIL</i>	4/55	7
<i>CD1D</i>	6/55	11
<i>GATA3</i>	2/55	4
<i>MYC</i>	0/55	0
<i>JUNB</i>	9/55	16
<i>TWIST1</i>	38/55	69
<i>EPHA4</i>	36/55	66
<i>STAT4</i>	50/55	91

Abbreviations: EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.

¹With the receiver operating characteristic curve analysis, a threshold was established at a specificity of 100% and an accuracy above 0.80. *PLS3*, *DNM3*, *TWIST1*, *EPHA4*, and *STAT4* were found to be useful diagnostic markers in SS.

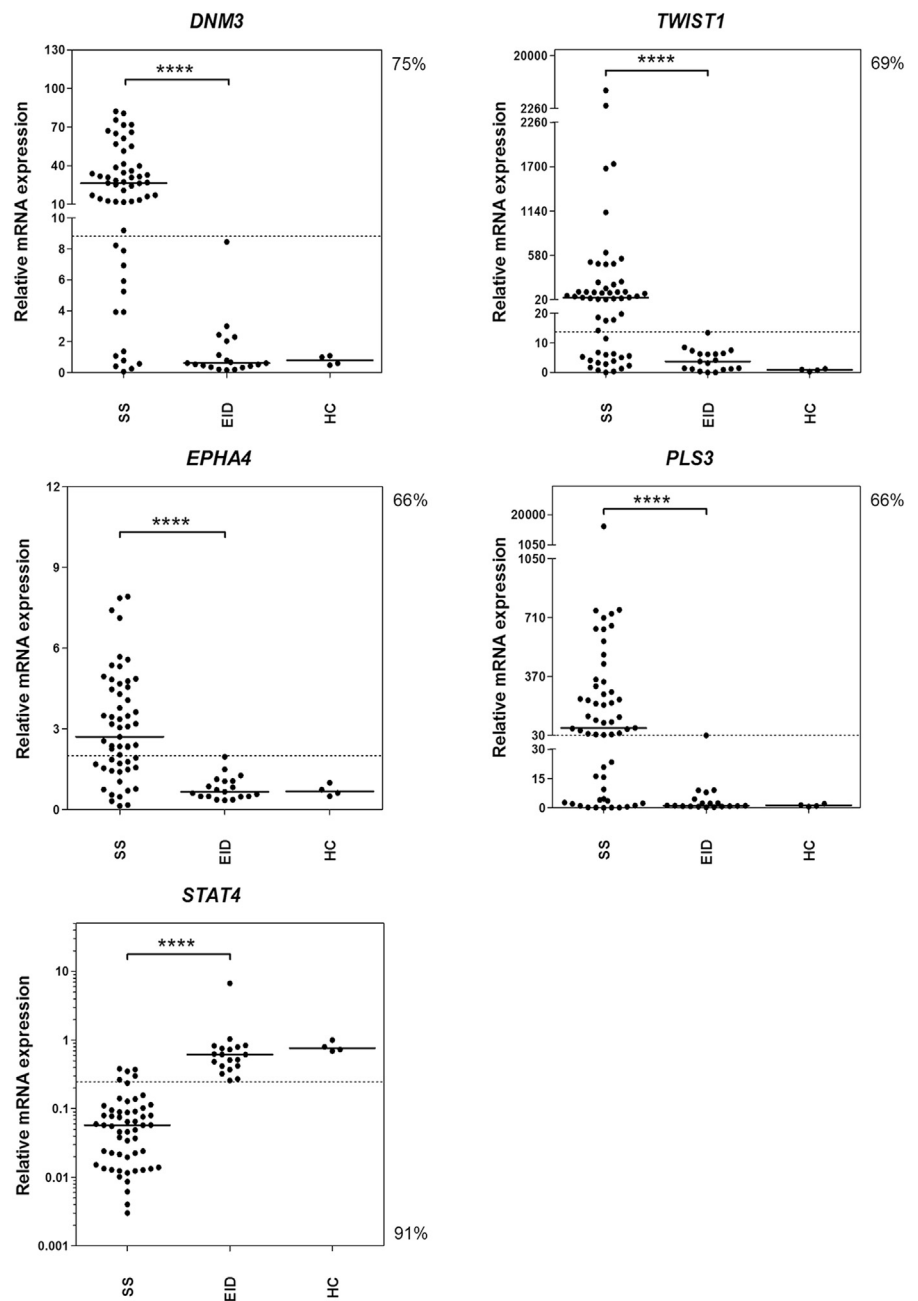
These discrepant results can be explained by different flow cytometry protocols. We evaluated CD26 expression on CD4⁺ gated T cells, whereas Bernengo et al. looked at CD4⁺CD26⁺ cells on gated total lymphocytes. Indeed, when looking at the CD4⁺CD26⁺ cells of 30% or more on total lymphocytes, similar results were found (sensitivity = 80%, specificity = 95%; data not shown).

A level of CD4⁺CD7⁺ cells of more than 40% has also been suggested as a tentative criterion in the diagnosis of SS (Vonderheid et al., 2002). Consistent with the literature, we found that loss of CD7 above 40% of the CD4⁺ T cells is highly specific (specificity = 100%) but is not a sensitive marker (sensitivity = 54%) (Harmon et al., 1996; Nagler et al., 2012; Vonderheid and Bernengo, 2003). Similar results were found for 40% or more CD4⁺CD7⁺ cells on total lymphocytes (sensitivity = 42%, specificity = 100%; data not shown).

Flow cytometry results show that in 83% of SS patients, CD4⁺ T cells display loss of CD26 by more than 80% and/or loss of CD7 by more than 40%, whereas this was never observed in EID patients. These observations are relevant because they can readily be included in immunophenotypic testing of erythrodermic patients.

Previous studies reported expression of killer cell immunoglobulin-like receptor CD158k in 65–97% of SS patients (Bahler et al., 2008; Klemke et al., 2008; Poszepczynska-Guigne et al., 2004). Flow cytometry analysis, performed in both Leiden and Paris, showed expression of CD158k in only 33% of SS patients (19 of 58), and in most of these SS patients (18 of 19) the CD158k antigen was expressed at low levels. This discrepancy can be explained by

Figure 2. Gene expression results for *DNM3*, *TWIST1*, *EPHA4*, *PLS3*, and *STAT4*. The differential gene expression is shown as relative normalized messenger RNA levels in 55 Sézary syndrome patients compared with 19 patients with erythrodermic inflammatory dermatoses and 4 healthy control subjects. ****Statistically significant difference in gene expression in Sézary syndrome compared with erythrodermic inflammatory dermatoses and healthy control subjects ($P < 0.001$). The dotted lines represent the thresholds for differential expression, determined with receiver operating characteristic curves with a specificity of 100%. The y-axis represents the relative messenger RNA expression with varying scale in all figures. EID, erythrodermic inflammatory dermatoses; HC, healthy control; mRNA, messenger RNA; SS, Sézary syndrome.



the fact that the present study was performed on frozen peripheral blood mononuclear cells (PBMCs) instead of freshly isolated PBMCs. Indeed, a recent study on freshly isolated PBMCs from SS patients showed high CD158k expression in Sézary cells (Moins-Teisserenc et al., 2015).

For CNV and GE analysis the use of SOPs and custom-made PCR platforms led to highly reproducible results as well. Gain of *MYC* and/or loss of its antagonist *MNT* was found in 76% of SS patients but never in EID patients. Gain of *TWIST1* and *JUNB* was detected in only a small minority of SS patients.

In line with the literature, we found up-regulation of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3* and down-regulation of *STAT4* in most SS patients (Boonen et al., 2008; Goswami et al., 2012; Kari et al., 2003; Nebozhyn et al., 2006; Su et al., 2003; Van Doorn R. et al., 2004). In contrast, only a minority of SS

patients showed up-regulation of *GATA3*, *CD1D*, *TRAIL*, *CDO1*, *JUNB*, and *MYC*, implying that these genes are not useful diagnostic markers. Why gain of *MYC* and loss of *MNT*, which is observed in most patients, does not lead to up-regulation of *MYC* expression is as yet unexplained.

Combined alterations in gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* could distinguish 98% of SS patients from EID patients, suggesting that this diagnostic panel will be useful as an additional molecular criterion in the diagnostic differentiation between SS and EID.

In the present study 27 patients were diagnosed with SS before inclusion in the study. However, no significant differences were found in the prevalence of the previously described markers between the 27 patients already diagnosed with SS before inclusion in the study and the 32 SS patients newly diagnosed at time of inclusion

(see [Supplementary Table S1](#) online). Similarly, the prevalence of investigated markers was similar in the 36 SS patients who received treatment during sample collection at inclusion and the 23 SS patients who did not receive any form of treatment (see [Supplementary Table S2](#) online). These observations argue that the observed immunophenotypic and molecular changes are stably expressed in Sézary cells.

We show that standardization of flow cytometry, CNV, and GE procedures leads to strong reproducibility of results. We argue that to facilitate comparison of results from different centers, it will be important to closely define the subset of cells that was investigated, and based on the present study we suggest gating on CD4⁺ T cells in future studies.

For patients in whom the distinction between SS and EID still cannot be made using the current diagnostic criteria, we propose that these two additional diagnostic panels can be used: (i) loss of CD26 ($\geq 80\%$ CD4⁺ T cells) and/or loss of CD7 ($\geq 40\%$ CD4⁺ T cells) for immunophenotypic analysis and (ii) combination of altered gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* for molecular analysis.

MATERIALS AND METHODS

Design of the study

To achieve sufficient power for the study, a consortium of six European Organization for Research and Treatment of Cancer centers with extensive experience with SS was formed including centers from Helsinki, Finland; London, England; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France. At time of inclusion peripheral blood samples were collected for investigation of (i) expression of cell surface proteins by flow cytometry, (ii) CNV, and (iii) GE profiles. The markers were selected based on the literature and are presented in [Supplementary Table S3](#) online.

To optimize standardization and to prevent interdepartmental differences, SOPs were produced for the workflow of blood sampling, isolation of PBMCs, and enrichment for CD4⁺ T cells (SOP 001), DNA isolation (SOP 002), RNA isolation (SOP 003), complementary DNA synthesis (SOP 004), CNV and GE assays (SOP 005), flow cytometry experiments (SOP 007), and the freezing and shipment of samples (SOP 008) (see [Supplementary Figure S4](#) and [Supplementary Materials](#) online). Much effort was put into standardizing flow cytometry analysis, because this technique has been shown to have limited reproducibility in multicenter studies because of limited standardization of laboratory procedures, instrumental settings, and interpretation of results ([van Dongen et al., 2012](#); [Westers et al., 2012](#)).

To test if the use of SOPs leads to increased reproducibility, the flow cytometry experiments were performed in duplicate in Leiden and Paris on all samples, and assays for CNV and GE were performed in Leiden and repeated in London for a selected number of samples.

In all participating centers the study was approved by the local institutional ethical review boards, and written informed patient consent was obtained. Consensus meetings to compare experimental results were held on August 31, 2012, in Paris and October 31, 2013, in Leiden.

Patient selection and clinical characteristics

Between September 2009 and October 2013 a total of 103 subjects were enrolled with the following diagnosis: SS ($n = 72$), EID ($n = 27$), and healthy controls ($n = 4$).

Inclusion criteria for the SS patients were diagnosis of SS based on the recent World Health Organization-European Organization for Research and Treatment of Cancer criteria and available complete clinical data. Inclusion criteria for patients with EID were presentation with erythroderma and blood test results not meeting the SS blood criteria.

From the initial 72 patients with SS 13 were excluded because of inferior sample quality ($n = 10$) or insufficient clinical data ($n = 3$). From the initial 27 EID patients eight were excluded because of inferior sample quality ($n = 3$) or insufficient clinical data ($n = 5$).

The final study group consisted of 59 patients with SS, 19 patients with EID, and four healthy controls. The SS group consisted of 32 patients with newly diagnosed SS and 27 patients with known SS. Thirty-six SS patients received treatment at the time of blood sampling (10 newly diagnosed with SS and 26 with known SS). The treatment consisted of psoralen plus UVA therapy ($n = 2$), extracorporeal photopheresis as monotherapy or combined with immunomodulatory agents ($n = 12$), prednisone alone or in combination with chlorambucil ($n = 9$), monotherapy with interferon alfa, bexarotene, methotrexate, or acitretin ($n = 11$), and polychemotherapy ($n = 2$).

The EID group included nine patients with atopic erythroderma, five patients with erythrodermic psoriasis, two patients with erythrodermic drug eruption, two patients with idiopathic erythroderma, and one patient with paraneoplastic erythroderma secondary to a cholangiocarcinoma. None of the EID patients developed a lymphoma during follow-up study (median follow-up = 22 months, range = 8–38 months).

Workup blood samples

PBMCs were isolated from peripheral blood and stored in liquid- or vapor-phase nitrogen. Part of the fresh PBMCs were enriched for CD4⁺ T helper cells by depletion of non-CD4⁺ T cells, resulting in greater than 95% purity for the CD4⁺ T-cell population, using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for CNV and GE assays.

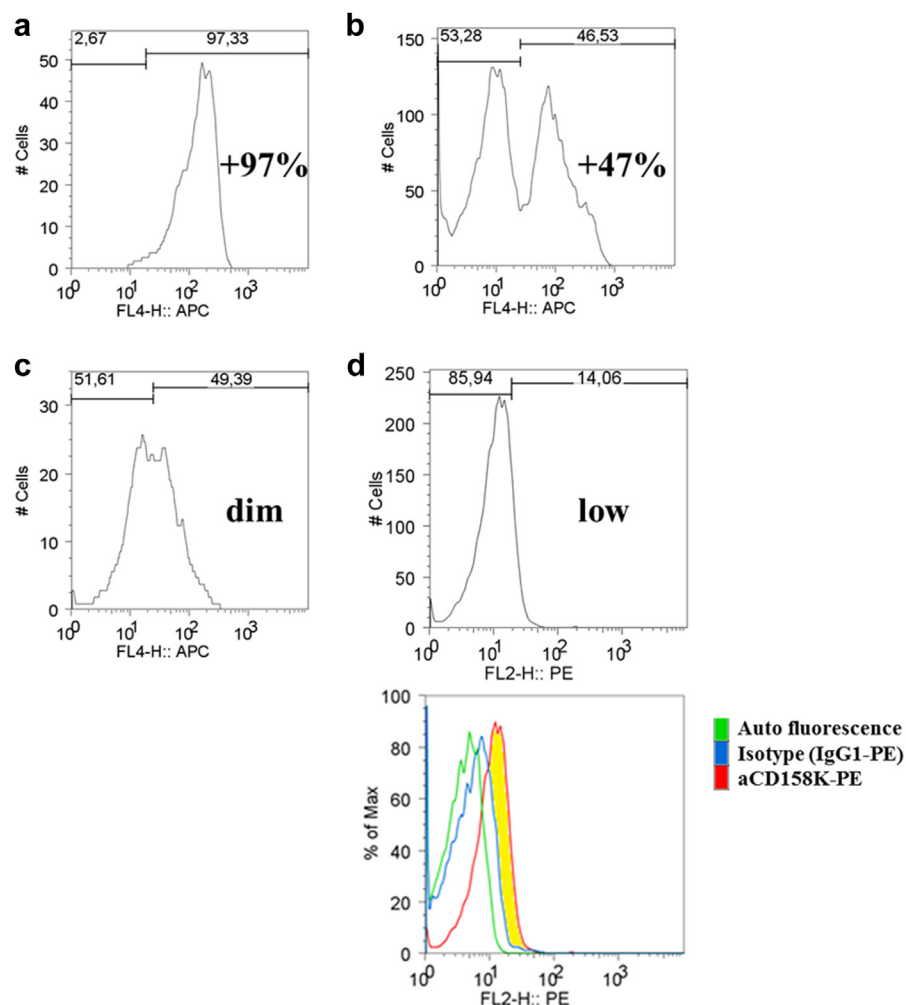
Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at -20°C .

RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. Two μg of RNA was reverse-transcribed in triplicate with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA), using random priming in a final volume of 20 μL . After synthesis, complementary DNA samples were stored at -20°C . A detailed description of this workup of blood samples is found in the [Supplementary Materials](#) (SOPs 001–004 and 008).

Flow cytometry

In Leiden and Paris flow cytometry was performed for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD27, CD45RA, CD45RO, CD158a, CD158b, and CD158k, including isotype-specific controls (see [Supplementary Table S4](#) online and SOP 007 in the [Supplementary Materials](#)). Lymphocytes were gated from forward and sideward scatter patterns; next, antigen expression was assayed from CD4⁺ gated lymphocytes. Specific antigen expression was observed relative to autofluorescence and (non-) specific signals obtained from each individual patient and a PBMC control sample. Specific antigen expression was observed relative to autofluorescence and (non-) specific signals, as determined with unlabelled cells, isotype-specific control and epitope-specific labelling relatively, from each individual patient and a PBMC control

Figure 3. Interpretation of flow cytometry results. (a) A single peak located on the right side of the determined threshold represents a single population with positive staining for CD7 in 97% of the gated cells. (b) Two distinct populations of the gated cells, in which 47% of the cells show positive expression for CD7 and 53% show negative staining. (c) Diminished expression for CD7 of a single population of gated cells, surrounding a determined threshold, is indicated as dim. (d). The specific CD158k antigen expression signal (indicated in red) is slightly shifted to the right compared with its autofluorescence and isotype control signals (green and blue lines), indicated in yellow. This shows that the gated cells do express CD158k but at very low level, indicated as “low.”



sample. This control sample was derived from two healthy donors and functioned as an internal reference sample for each flow cytometry session. Samples were analyzed in a blinded setting.

Specific antigen expression in the population of gated cells (expression or loss) was displayed in percentages (Figure 3a and b). Antigen expression was considered dim if all gated cells showed diminished expression around the determined threshold (Figure 3c). For CD158a, CD158b, and CD158k, expression below 5% of the gated cells was considered as no expression, but when intermediate expression of a single population of gated cells, surrounding a determined threshold, was found, this was characterized as low-expressing antigen (for example, CD158k^{low}) (Figure 3d).

CNV assay

Quantitative PCR assays with 6-Carboxyfluorescein-labeled hydrolysis Minor Groove Binder probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, and *MNT* and reference genes *ABT1*, *ARG2*, and *DNM3* (see Supplementary Table S5 online). Reference genes were selected from different large copy number-stable chromosomal regions in SS, selected from array-based comparative genomic hybridization experiments on 20 SS samples (Vermeer et al., 2008). Amplification efficiency was evaluated in triplicate, using eight 4-fold serial dilution points ranging from 3 ng/μL to 183 fg/μL DNA concentration, under optimized primer and hydrolysis probe concentrations. Assays with amplification efficiency value between 90% and 100% and a

correlation coefficient above 0.98 were accepted for CNV analysis. Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005 (see Supplementary Materials).

Data were normalized against reference genes and relative to the common reference using the $\Delta\Delta C_q$ method and are presented as relative copy number, where 2 stands for diploid DNA (Livak and Schmittgen, 2001). The following thresholds were maintained for the CNV data: 1.5–2.5 was considered as diploid (normal) DNA, greater than 2.5 as gain in copy number, and less than 1.5 as loss in copy number.

GE assay

GE quantitative PCR assays with FAM-labelled hydrolysis MGB probes (Life Technologies) were developed and validated, as described for CNV quantitative PCR assays, for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, and *STAT4* and reference genes *ARF5*, *ERCC3*, and *TMEM87A* (see Supplementary Table S5). Stably expressed reference genes were selected from microarray experiments on SS samples and validated in SS and EID samples according to the GeNorm method (Boonen et al., 2008; Vandesompele et al., 2002; Van Doorn R. et al., 2004). Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005. Receiver operating characteristic curve analyses were used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. A one-tailed

Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant.

CONFLICT OF INTEREST

MF received travel grants from TEVA and ICON.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.01.038>.

REFERENCES

- Bagot M, Moretta A, Sivori S, Biassoni R, Cantoni C, Bottino C, et al. CD4(+) cutaneous T-cell lymphoma cells express the p140-killer cell immunoglobulin-like receptor. *Blood* 2001;97:1388–91.
- Bahler DW, Hartung L, Hill S, Bowen GM, Vonderheid EC. CD158k/KIR3DL2 is a useful marker for identifying neoplastic T-cells in Sezary syndrome by flow cytometry. *Cytometry B Clin Cytom* 2008;74:156–62.
- Bernengo MG, Novelli M, Quaglino P, Lisa F, De Matteis A, Savoia P, et al. The relevance of the CD4+ CD26- subset in the identification of circulating Sézary cells. *Br J Dermatol* 2001;144:125–35.
- Booken N, Gratchev A, Utikal J, Weiss C, Yu X, Qadoumi M, et al. Sezary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNMT3. *Leukemia* 2008;22:393–9.
- Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. *Blood* 2010;116:767–71.
- Dummer R, Heald PW, Nestle FO, Ludwig E, Laine E, Hemmi S, et al. Sezary syndrome T-cell clones display T-helper 2 cytokines and express the accessory factor-1 (interferon-gamma receptor beta-chain). *Blood* 1996;88:1383–9.
- Duncan SC, Winkelmann RK. Circulating Sézary cells in hospitalized dermatology patients. *Br J Dermatol* 1978;99:171–8.
- Fierro MT, Novelli M, Quaglino P, Comessatti A, Fava P, Ortoncelli M, et al. Heterogeneity of circulating CD4+ memory T-cell subsets in erythrodermic patients: CD27 analysis can help to distinguish cutaneous T-cell lymphomas from inflammatory erythroderma. *Dermatology* 2008;216:213–21.
- Goswami M, Duvic M, Dougherty A, Ni X. Increased Twist expression in advanced stage of mycosis fungoides and Sezary syndrome. *J Cutan Pathol* 2012;39:500–7.
- Harmon CB, Witzig TE, Katzmman JA, Pittelkow MR. Detection of circulating T cells with CD4+CD7- immunophenotype in patients with benign and malignant lymphoproliferative dermatoses. *J Am Acad Dermatol* 1996;35:404–10.
- Jones D, Dang NH, Duvic M, Washington LT, Huh YO. Absence of CD26 expression is a useful marker for diagnosis of T-cell lymphoma in peripheral blood. *Am J Clin Pathol* 2001;115:885–92.
- Karenko L, Nevala H, Raatikainen M, Franssila K, Ranki A. Chromosomally clonal T cells in the skin, blood, or lymph nodes of two Sezary syndrome patients express CD45RA, CD45RO, CDw150, and interleukin-4, but no interleukin-2 or interferon-gamma. *J Invest Dermatol* 2001;116:188–93.
- Kari L, Loboda A, Nebozhyn M, Rook AH, Vonderheid EC, Nichols C, et al. Classification and prediction of survival in patients with the leukemic phase of cutaneous T cell lymphoma. *J Exp Med* 2003;197:1477–88.
- Kelemen K, Guitart J, Kuzel TM, Goolsby CL, Peterson LC. The usefulness of CD26 in flow cytometric analysis of peripheral blood in Sezary syndrome. *Am J Clin Pathol* 2008;129:146–56.
- Klemke CD, Brade J, Weckesser S, Sachse MM, Booken N, Neumaier M, et al. The diagnosis of Sezary syndrome on peripheral blood by flow cytometry requires the use of multiple markers. *Br J Dermatol* 2008;159:871–80.
- Lima M, Almeida J, dos Anjos TM, Queiros ML, Santos AH, Fonseca S, et al. Utility of flow cytometry immunophenotyping and DNA ploidy studies for diagnosis and characterization of blood involvement in CD4+ Sezary's syndrome. *Haematologica* 2003;88:874–87.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8.
- Mao X, Orchard G, Lillington DM, Russell-Jones R, Young BD, Whittaker SJ. Amplification and overexpression of JUNB is associated with primary cutaneous T-cell lymphomas. *Blood* 2003;101:1513–9.
- Mao X, Orchard G, Mitchell TJ, Oyama N, Russell-Jones R, Vermeer MH, et al. A genomic and expression study of AP-1 in primary cutaneous T-cell lymphoma: evidence for dysregulated expression of JUNB and JUND in MF and SS. *J Cutan Pathol* 2008;35:899–910.
- Marie-Cardine A, Huet D, Ortonne N, Remtoul N, Le GS, Bagot M, et al. Killer cell Ig-like receptors CD158a and CD158b display a coactivatory function, involving the c-Jun NH2-terminal protein kinase signaling pathway, when expressed on malignant CD4+ T cells from a patient with Sezary syndrome. *Blood* 2007;109:5064–5.
- Meijer CJ, van Leeuwen AW, van der Loo EM, van de Putte LB, van Vloten WA. Cerebriform (Sezary like) mononuclear cells in healthy individuals: a morphologically distinct population of T cells. Relationship with mycosis fungoides and Sezary's syndrome. *Virchows Arch B Cell Pathol* 1977;25:95–104.
- Michel L, Jean-Louis F, Begue E, Bensussan A, Bagot M. Use of PLS3, Twist, CD158k/KIR3DL2, and Nkp46 gene expression combination for reliable Sezary syndrome diagnosis. *Blood* 2013;121:1477–8.
- Moins-Teisserenc H, Daubord M, Clave E, Douay C, Felix J, Marie-Cardine A, et al. CD158k is a reliable marker for diagnosis of Sezary syndrome and reveals an unprecedented heterogeneity of circulating malignant cells. *J Invest Dermatol* 2015;135:247–57.
- Nagler AR, Samimi S, Schaffer A, Vittorio CC, Kim EJ, Rook AH. Peripheral blood findings in erythrodermic patients: importance for the differential diagnosis of Sezary syndrome. *J Am Acad Dermatol* 2012;66:503–8.
- Nebozhyn M, Loboda A, Kari L, Rook AH, Vonderheid EC, Lessin S, et al. Quantitative PCR on 5 genes reliably identifies CTCL patients with 5% to 99% circulating tumor cells with 90% accuracy. *Blood* 2006;107:3189–96.
- Olsen E, Vonderheid E, Pimpinelli N, Willemze R, Kim Y, Knobler R, et al. Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood* 2007;110:1713–22.
- Poszepczynska-Guigne E, Schiavon V, d'Incan M, Echchakir H, Musette P, Ortonne N, et al. CD158k/KIR3DL2 is a new phenotypic marker of Sezary cells: relevance for the diagnosis and follow-up of Sezary syndrome. *J Invest Dermatol* 2004;122:820–3.
- Rappl G, Muche JM, Abken H, Sterry W, Tilgen W, Ugurel S, et al. CD4(+) CD7(-) T cells compose the dominant T-cell clone in the peripheral blood of patients with Sezary syndrome. *J Am Acad Dermatol* 2001;44:456–61.
- Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986;15:1217–26.
- Sokolowska-Wojdylo M, Wenzel J, Gaffal E, Steitz J, Roszkiewicz J, Bieber T, et al. Absence of CD26 expression on skin-homing CLA+ CD4+ T lymphocytes in peripheral blood is a highly sensitive marker for early diagnosis

- and therapeutic monitoring of patients with Sezary syndrome. Clin Exp Dermatol 2005;30:702–6.
- Su MW, Dorocicz I, Dragowska WH, Ho V, Li G, Voss N, et al. Aberrant expression of T-plastin in Sezary cells. Cancer Res 2003;63:7122–7.
- Swerdlow SH, Campo E, Lee Harris N, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. Geneva, Switzerland: WHO Press; 2008. p. 299.
- Trotter MJ, Whittaker SJ, Orchard GE, Smith NP. Cutaneous histopathology of Sezary syndrome: a study of 41 cases with a proven circulating T-cell clone. J Cutan Pathol 1997;24:286–91.
- Van Dongen JJ, Orfao A, EuroFlow Consortium: EuroFlow. Resetting leukemia and lymphoma immunophenotyping. Basis for companion diagnostics and personalized medicine. Leukemia 2012;26:1899–907.
- Van Doorn R, Dijkman R, Vermeer MH, Out-Luiting JJ, van der Raaij-Helmer EM, Willemze R, et al. Aberrant expression of the tyrosine kinase receptor EphA4 and the transcription factor twist in Sezary syndrome identified by gene expression analysis. Cancer Res 2004;64: 5578–86.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 2002;3.
- Vermeer MH, van Doorn R, Dijkman R, Mao X, Whittaker S, van Voorst Vader PC, et al. Novel and highly recurrent chromosomal alterations in Sezary syndrome. Cancer Res 2008;68:2689–98.
- Vonderheid EC. On the diagnosis of erythrodermic cutaneous T-cell lymphoma. J Cutan Pathol 2006;1(Suppl. 33):27–42.
- Vonderheid EC, Bernengo MG. The Sezary syndrome: hematologic criteria. Hematol Oncol Clin North Am 2003;17:1367–89.
- Vonderheid EC, Bernengo MG, Burg G, Duvic M, Heald P, Laroche L, et al. Update on erythrodermic cutaneous T-cell lymphoma: report of the International Society for Cutaneous Lymphomas. J Am Acad Dermatol 2002;46: 95–106.
- Washington LT, Huh YO, Powers LC, Duvic M, Jones D. A stable aberrant immunophenotype characterizes nearly all cases of cutaneous T-cell lymphoma in blood and can be used to monitor response to therapy. BMC Clin Pathol 2002;2:5.
- Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P, et al. Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. Leukemia 2012;26:1730–41.
- Wieselthier JS, Koh HK. Sezary syndrome: diagnosis, prognosis, and critical review of treatment options. J Am Acad Dermatol 1990;22:381–401.
- Willemze R, Kerl H, Sterry W, Berti E, Cerroni L, Chimenti S, et al. EORTC classification for primary cutaneous lymphomas: a proposal from the Cutaneous Lymphoma Study Group of the European Organization for Research and Treatment of Cancer. Blood 1997;90:354–71.
- Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, et al. WHO-EORTC classification for cutaneous lymphomas. Blood 2005;105: 3768–85.